Microbiome differentiation between ant castes implicates new microbial roles in the fungus-growing ant *Trachymyrmex septentrionalis*

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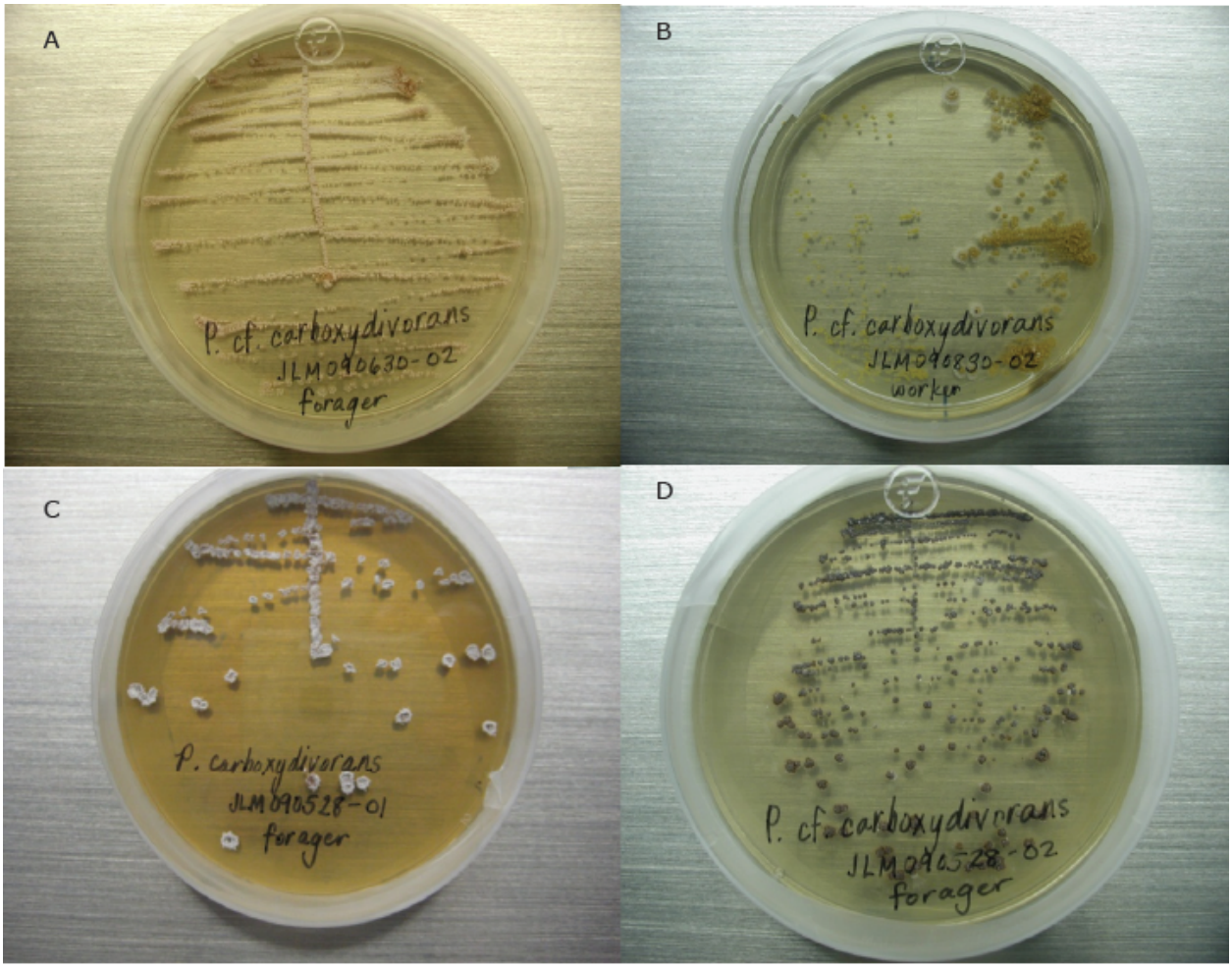
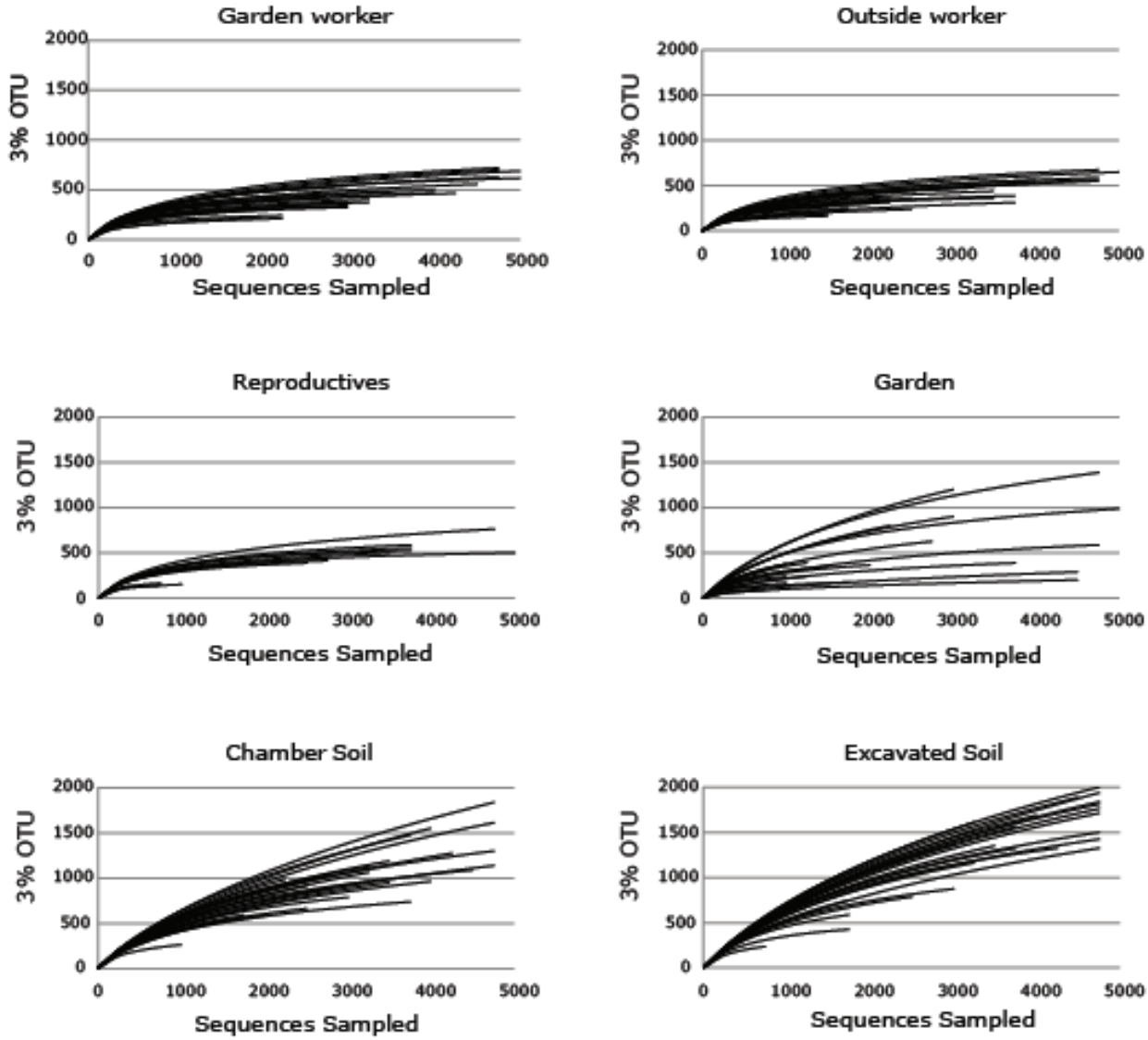
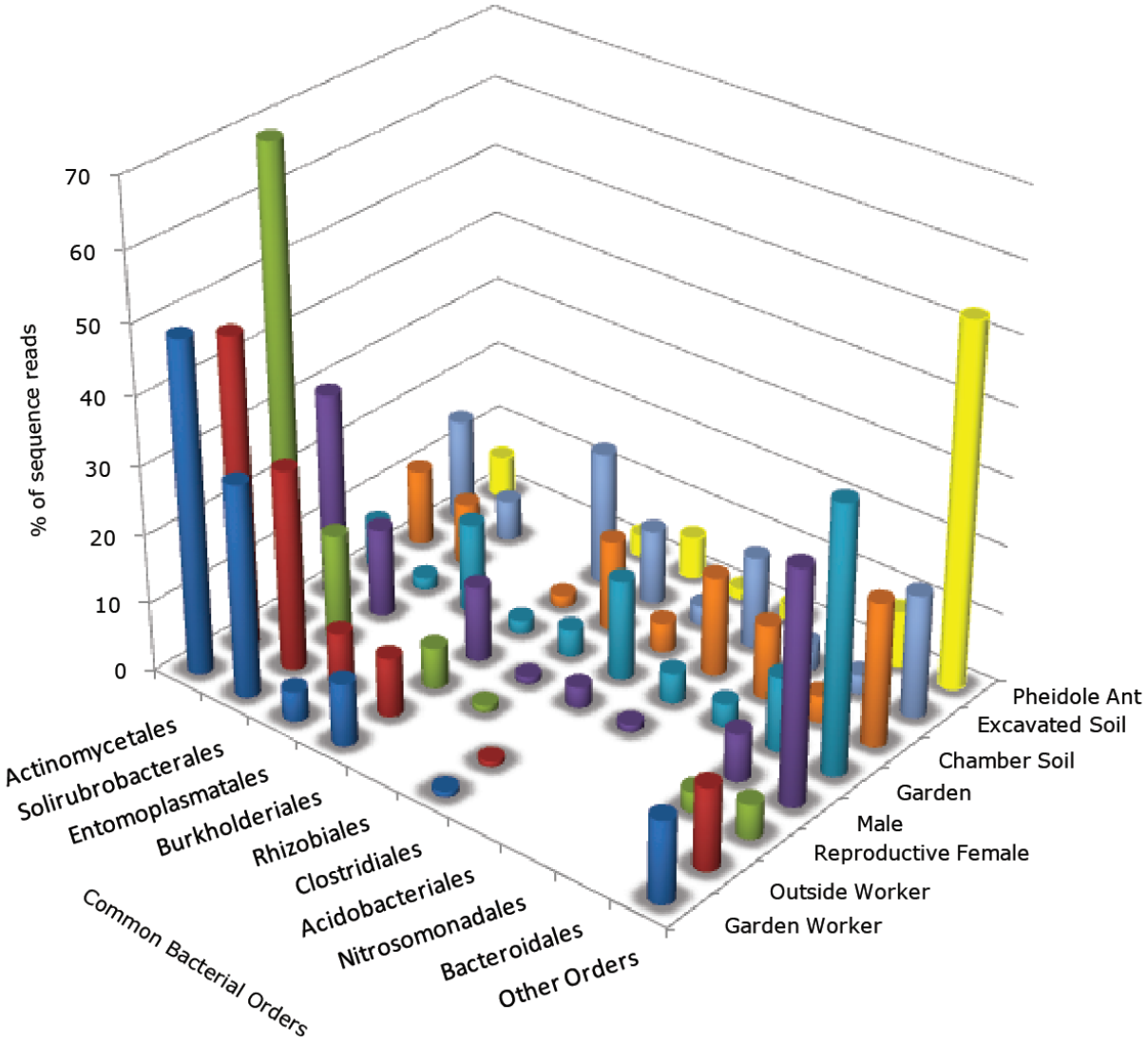
Supplementary Figure S1. *Pseudonocardia cf. carboxydivorans* morphotypes: (A) light tan, circular growth (B) yellow, circular growth (C) white, irregular growth front (D) dark brown, circular growth. Despite the range of morphology, all of the isolates shared identical partial 16S sequences.



Supplementary Figure S2. Rarefaction analyses of bacterial Operational Taxonomic Units (OTUs) associated with *Trachymyrmex septentrionalis*. OTUs are binned at 3% sequence dissimilarity. Samples from garden workers, outside workers, and reproductives (male and females) appear to reach an asymptote at 1000-5000 sequences sampled, but, as expected, soil samples require much greater sequencing depth (probably more than 10,000-100,000 sequences) to profile the full bacterial diversity. Some of the garden samples reach an asymptote with a sequencing depth between 1000-5000, but bacterial diversity in other gardens was under-sampled.



Supplementary Figure S3. Relative abundances or percent sequence reads of common bacterial orders associated with *T. septentrionalis* ants, its nest environment, and *Pheidole* control ants collected nearby. Relative abundances are percentages calculated by counting the 454-sequences identified to order by BLAST match. Total sequence reads were averaged for each sample type (garden workers, outside workers, reproductive females, males, garden, chamber soil, excavated soil, and *Pheidole* ants). Two orders, Actinomycetales and Solirubrobacterales (both in the class Actinobacteria), had the most sequence reads associated with *T. septentrionalis* garden workers, outside workers, males, and reproductive females. *Pheidole* ants show very different bacterial profiles compared to *Trachymyrmex* ants (common bacteria for *Pheidole* ants are listed in Tables S1 and S2).



Supplementary Table S1. BLAST match to closest taxonomic identity. 454-sequences were identified to their closest taxonomic level from BLAST hits using a high quality 16S reference database curated by the Medical Biofilm Institute. The reference BLAST-assignments are presented according to percent sequence-identity to particular taxonomic levels (i.e., sequences with a greater than 97% sequence-identity match were resolved to species: between 95-97% to genus, between 90-95% to family, between 85- 90% to order, 80-85% to class, and 75-80% to phylum).

Supplementary Table S2. BLAST results to nearest forced genus.

BLAST was used to identify raw 454 sequence tags to a reference sequence from the Medical Biofilm Research Institute 16S database. This table presents the data forced to the nearest bacterial genus (matched at a 100- 75% hit identity) with an average blast hit having a 93.8% (+/- 4.2) identity match. This forced BLAST table was not intended to be a strict reference assignment, but was useful to compare the variation of sequence reads observed within samples.

Supplementary Table S3. Bacterial genera found in ants and soils per nest. To evaluate possible ecological links between ant-associated microbes and microbes in the soil, we counted the number of shared bacterial genera identified in ant samples (not including the garden) and soil samples of the same nest. 65% of the bacterial genera found in the ant samples were shared with the bacterial genera found in the soil, suggesting possible ecological connectivity between bacterial communities associated with ants and with soil.

Nest	# Bacterial Genera in Ants	# Bacterial Genera in Soils	Genera Shared between Ants & Soil	% Shared/Ant	% Shared/Soil
J0201	63	180	37	58.7	20.6
J0303	78	255	58	74.4	22.7
J0304	55	277	40	72.7	14.4
J032A	133	227	79	59.4	34.8
J032B	151	359	123	81.5	34.3
J0401	82	263	59	72.0	22.4
J0402	106	247	64	60.4	25.9
J0403	103	275	58	56.3	21.1
J0404	78	272	59	75.6	21.7
J0501	87	248	62	71.3	25
J0502	63	308	41	65.1	13.3
J0503	77	318	50	64.9	15.7
J0504	99	292	76	76.8	26
J0601	82	295	57	69.5	19.3
J0602	88	210	58	65.9	27.6
J0603	65	262	44	67.7	16.8
J0630-03	73	226	38	52.1	16.8
J0701	96	181	43	44.8	23.8
J0702	80	185	39	48.8	21.1
J0704	113	216	69	61.1	31.9
J0801	78	372	59	75.6	15.9
J0802	46	245	28	60.9	11.4
J0803	103	215	49	47.6	22.8
J1-7	145	260	106	73.1	40.8
J8-12	68	280	49	72.1	17.5
Average	**88.5**	**258.7**	**57.8**	**65.1**	**22.5**
Standard Deviation	**26.3**	**49.5**	**21.2**		

Supplementary Table S4. BLAST results according to two 16S reference databases. Sample subset comparison of BLAST results using two different 16S reference databases: Ribosomal Database Project (RDP) Classifier and a high-quality 16S database curated by the Medical Biofilm Research Institute (MBRI). We performed a BLAST on all of the 454-sequences using the two reference databases, but a subset of the results are shown in the table from collection months February and June. This table illustrates some of the differences between BLAST hits from two different reference databases. Both databases identified the most abundant genera found in all samples as *Solirubrobacter* and *Pseudonocardia* with a 93% and 98% similarity, respectively. However, much of the rare bacterial strains did not match between the databases. Overall, this comparison served to increase our confidence in the common bacteria, but any rare genera results should be interpreted with caution.

Supplementary Table S5. Abundance of *Pseudonocardia* strains of *T. septentrionalis* and their phylogenetic placement. Hits from a custom BLAST of *Pseudonocardia* 454-sequences to a *Pseudonocardia* reference database were grouped for each sample type into the 10 subclades of *Pseudonocardia* identified previously in the phylogenetic analysis of [1]. Total number of different sequences (second to last row) indicates the number of *Pseudonocardia* sequences generated by 454-sequencing. Because some of the 454 sequences BLAST to the same reference sequence in the *Pseudonocardia* phylogeny, we also list the total number of distinct strains placed into each clade, defined as the number of unique BLAST hits to one of the 116 sequences included in our *Pseudonocardia* database. BLAST hits for all 26,965 sequences are shown in Supplementary Table S8. The genus *Pseudonocardia* is split basally into two main subgroups, one containing clades 1-5, the other containing clades 6-10 [1]. Although all ant samples combined (garden worker, outside worker, reproductive females, and males) carried *Pseudonocardia* from most of the ten clades (except strains from clades 2, 4, and 5 were not found on ants), the majority of the ant-associated *Pseudonocardia* sequences were placed into clade 3 (the so-called *nitrificans/alni/carboxydivorans* clade *sensu* [1]). The garden and soil samples also contained *Pseudonocardia* from almost all the clades, but the majority of these sequences are found in clades 6-10, which are *Pseudonocardia* types found more frequently in soil (see Fig. 2b in [1]).

	Garden Worker		Outside Worker		Reproductive Female		Male		Garden		Chamber soil		Excavated Soil		*Pheidole* An	
amples creened	25		25		9		3		22		25		24		4	
lades Name	seq	strains	seq	strains	seq	strains	seq	strains	seq	strains	seq	strains	seq	strains	seq	strain
lade 1	4	3	3	2	0	0	0	0	0	0	47	5	25	6	0	0
lade 2	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
lade 3	7,281	3	2,879	3	12,070	3	2,088	2	50	1	13	1	3	1	0	0
lade 4	0	0	0	0	0	0	0	0	2	1	1	1	3	1	0	0
lade 5	0	0	0	0	0	0	0	0	0	0	2	2	8	3	0	0
lade 6	4	2	18	3	3	1	2	1	98	7	348	9	243	8	0	0
lade 7	7	2	3	1	1	1	0	0	0	0	28	3	1	1	0	0
lade 8	2	1	2	1	0	0	2	2	20	2	463	9	319	8	2	1
lade 9	8	2	2	2	0	0	0	0	5	4	44	6	131	6	0	0
rade between lades 9 and 10	2	1	6	2	0	0	0	0	302	4	172	7	220	6	0	0
lade 10	0	0	0	0	1	1	0	0	2	2	10	2	14	3	0	0
lumber of 454-sequences generated	7,308		2,913		12,075		2,092		479		1,128		968		2	
Number of listinct strains among all sequences		14		14		6		5		21		45		44		1

Supplementary Table S6. 16S primers for amplification and sequencing of cultured isolates. Primer combinations used to amplify partial 16S rDNA sequences for culture-dependent 16S-sequence identification of isolated bacterial morphotypes.

Primer – Forward 5'-3'	Tm	Source	Primer – Reverse 5'-3'	Tm	Source	Bacterial genera that amplified with primer combination
8F - AGA GTT TGA TCC TGG CTC AG	61	[2]	897R_hdi- GGT AAG GTT CTT CGC GTT GC	66	Developed by H. Ishak	some *Streptomyces*
133F_hdi - GAGTAA CAC GTG GGY GAC CTG C	68	Developed by H. Ishak	897R_hdi- GGT AAG GTT CTT CGC GTT GC	66	Developed by H. Ishak	All bacteria from our survey except *Nonomuraea*.
261F_hdi - CTG GGA CTG AGA CAC GGC	65	Developed by H. Ishak	897R_hdi- GGT AAG GTT CTT CGC GTT GC	66	Developed by H. Ishak	All bacteria from our survey except *Nonomuraea*. Main primer set used.
AMP2- GTG GAA AGT TTT TTC GGC TGG GG	53	[3]	AMP3- GCG GCA CAG AGA CCG TGG AAT	53	[3]	*Kribbella, Nocardia, Pseudonocardia spinospora, P. carboxydivorans,* and some *Streptomyces*
U519F- CAG CMG CCG CGG TAA TWC	54	[4]	U1406R- GAC GGG CGG TGT GTR CA	52	[5]	*Kribbella, Nocardia, Nonomuraea,* and some *Streptomyces*
U519F_Psadj - CAG CWG CCG CGG TAA YAC	64	[4]	U1406R- GAC GGG CGG TGT GTR CA	52	[5]	*Pseudonocardia alni* and *P. carboxydivorans*

Supplementary Table S7. Raw output from the Medical Biofilm Research Institute BLAST. The BLAST output report includes the raw sequence read, the % identity score, and the genus reference assignments. The sequence identity scores range from 100-75% with higher scores indicating a better match.

Supplementary Table S8. Raw output for the *Pseudonocardia*-specific BLAST. The BLAST used a comprehensive *Pseudonocardia* reference database derived from the phylogenetic analysis in [1]

Supplementary Methods

The Study System

The fungus-growing ant *Trachymyrmex septentrionalis* is a suitable study system for a phenological survey of bacterial-community associations because (a) colony sizes are large enough to permit repeat sampling of single nests in the field, but small enough for easy sampling of an entire colony; (b) nests occur at high densities in most populations, so many nests can be studied in the same habitat; (c) nests occur in sandy soil, facilitating nest excavation; (d) nest architecture is simple, with 2-5 gardens total (mode of 2-3 gardens), topmost garden chambers are found in spring at a depth of 5-15 cm, and the deepest garden chambers almost never exceed 80cm depth in central Texas; (e) most importantly, colonies undergo an annual cycle where garden sizes are greatly reduced during winter (gardens are sometimes reduced to small fragments carried by a few workers; [6,7]), foraging ceases during the coldest months, gardens are reactivated in spring, and gardens reach the largest sizes throughout summer when alates are produced. *T. septentrionalis* is the only fungus-growing ant known with such extreme changes in garden size between seasons [6]. *T. septentrionalis* alates will wait in the nest until rains stimulate mating flights. The study site experienced drought conditions in 2009, according to nearby precipitation data from the National Climate Data Center for Austin Bergstrom public database (http://www.nws.noaa.gov/climate/index.php?wfo=ewx) there were only three days between June and September with >1" precipitation (July 22, August 12, and Aug 27), which may have stimulated mating flights.

Culture-dependent isolation

Samples for culture-dependent isolation were collected in vials containing 1-mL of autoclaved saline buffer (0.7g K_2HPO_4, 0.5g $MgSO_4$, 0.3g KH_2PO_4, 0.01g $FeSO_4$, 0.001g $ZnSO_4$ in 1 L

ultrapure water). Samples in saline vials were vortexed for 10 min to dislodge microbes, and then 50μL aliquots of the vortexed saline were spread on two replicate chitin plates containing a minimum-carbon medium that favors growth of autotrophic bacteria[8]. In addition, ants housed individually in sterile, blank vials (i.e. vials without ethanol or saline buffer) were separated into head, mesosoma, and metasoma using flame-sterilized forceps, and then each body segment was streaked directly onto chitin plates (streaking the body part over the medium surface). Growth of the first actinomycete colonies was visible on the chitin plates after 8-10 days incubation at room temperature. A subset of representative actinomycete colonies visible 7-14 days after inoculation were transferred to potato dextrose agar (PDA) and maintained as pure live cultures for morphotyping and 16S rDNA Sanger-sequencing. After 14 days, chitin plates generally became overgrown with contaminant fungi and isolation of actinomycete bacteria was terminated.

Identification of actinomycete morphotypes

Each actinomycete colony was morphotyped according to color and growth morphology on the PDA medium. The actinomycete morphotypes were each identified by sequencing a portion of the 16S rDNA gene. DNA was extracted from a small sample of actinomycete growth taken from a pure live culture using a standard 10% Chelex protocol (Sigma-Aldrich). A fragment of the 16S gene was amplified and then sequenced on an ABI 3100 automated sequencer (16S primers are listed in Supplementary Table S5). All primer pairs used the following PCR cycling profile: 94°C for 4 min; 35 cycles of 94°C for 1 min, 50°C for 1 min, 70°C for 2 min; final 72°C incubation for 10 min. 264 total sequences obtained from the isolated bacteria were assigned to genus or species in fall 2009 according to their closest hit via nucleotide BLAST at the Core Nucleotide Collection deposited in the National Center for Biotechnology Information (NCBI). Once we had identified at least three cultures of each morphotype through 16S sequencing (e.g.,

Amycolatopsis, Kribbella, Streptomyces, *Pseudonocardia*, etc.), the remaining cultures were classified by their respective morphotype appearance on PDA plates.

454 Sequencing Fast UniFrac Analyses

For community comparison analysis, we used a custom Perl script to randomly sub-sample 1000 sequences from each bacterial community sequenced. In four samples, less than 1000 sequences had been generated, so we used all the respective sequences from these four samples. Garden sample J0304-G had a failed 454-pyrosequencing run and therefore had to be excluded from the analyses. The randomly-sampled sequences were clustered by sequence similarity using the web-based program cd-hit-est [9] with a minimum identity of 97% within each cluster. The longest sequence read from each cluster was selected as a representative sequence for that cluster for further analysis. These representative sequences were aligned using the sequence pipeline in Mothur with the SILVA alignment as a template[10] (www.mothur.org). The final alignment consisted of 1,787 total sequences with an average sequence length of 445 base pairs (bp) and a range of 300 bp to 510 bp. An approximate maximum-likelihood phylogenetic tree was generated by FastTree [11]. We used Fast Unifrac [12] to assess the differences between the bacterial communities associated with ants, gardens, and the two types of soil sampled. UniFrac distances are based on the phylogenetic tree branch lengths shared between two communities. A large UniFrac distance between two communities implies that they are not similar, and therefore members of the compared bacterial communities tend to be more distantly related. We used an abundance-weighted principal coordinate analysis (PCoA) to evaluate differences in bacterial community composition. All 454-sequencing data can be found at NCBI in the short read archive (SRP008669) with the exception of samples J1-J12 whose .sff files were lost. A complete data set in fasta format can be requested from the authors.

List of sequences selected for the custom Pseudonocardia BLAST

116 sequences were used for the custom BLAST derived from the recently published global *Pseudonocardia* phylogenetic analysis [1]. These 116 sequences were chosen from the complete dataset [1] (n=334) by removing redundant sequences (i.e., sequences with at least 99.5% sequence similarity) and removing sequences that did not cover the complete V1-V3 region of the 16S gene. The below sequence names retain their original name as it appeared in [1], but the clade numbers were added for clarity. Note that respective GenBank accessions are incorporated within each taxon name.

>Clade1PyroSequGQ082333nbw1151a06c1humanskinUSA

>Clade1PyroSequGQ008081nbw113d01c1humanskinUSA

>Clade1PyroSequGQ009429nbw776e01c1humanskinUSA

>Clade1PendophyticaCulturedDQ887489YIM56035endophyteChina

>Clade1simtoPendophyticaCulturedEF216352TFS701fjordsedimentNorway

>Clade1PyroSequGQ002479nbu177h11c1humanskinUSA

>Clade1simtoPendophyticaCulturedX87314SR244aleaflitterAustralia

>Clade1CulturedAY376892ApdentigerumA38workerPanama

>Clade1CulturedFJ948117MysmithiiUGM01040103T1workerlabnestUSA

>Clade1CulturedAY944264S07marinespongeChinaSea

>Clade1PkongjuensisCulturedAJ252833LM157cavesoilSouthKorea

>Clade1simtoPammonioxydansCulturedEU925632JSM074014anemonesymbiontChina

>Clade1PammonioxydansCulturedAY500143H9AS41877coastalsedimentChina

>Clade1CulturedFJ490529Ao19Acoctospinosus

>Clade2PparietisCulturedFM86370304St002mouldywallGermany

>Clade2PficiCulturedEU200678YIM56250endophyteChina

>Clade3PtropicaspnovCulturedGQ906587YIM61452endophyteChina

>Clade3CulturedEF588222AcspSP03040501workerArgentina

>Clade3CulturedFJ490549Ao2AcoctospinosusPanama

>Clade3PnitrificansCulturedNEWGENBANKNRRLB1664soilUSA

>Clade4PacaciaeEU921261GMKU095plantrootThailand

>Clade4CulturedFJ805426EUM374endophyteAustralia

>Clade5CulturedFJ948115CywheeleriUGM03042701Y1workerlabnest

>Clade5CulturedAJ007000LAA2compostbiofilterCanada

>Clade5CulturedAF131480IM6067rainforestsoilSingapore

>Clade5PailaonensisCulturedDQ344632YIM45505soilChina

>Clade5CulturedAJ006999LAA1compostbiofilterCanada

>Clade5CulturedFJ817397YIM63638endophyteChina

>Clade5PhalophobicaCulturedAJ252827IMSNU21327TypeStrainsoil

>Clade5PhalophobicaCulturedGQ179660S4201endophyteThailand

>Clade6PbabensisspnovCulturedAB514449VN05A0561plantlitterVietnam

>Clade6CulturedspEU722523S053sourceunknown

>Clade6ClonedAJ400508Hb1K67deterioratingpaintingAustria

>Clade6CulturedDQ344633YIM45552

>Clade6PxinjiangensisCulturedEU722520XJ45TypeStrainsoilChina

>Clade6CulturedEU81088001Q8ScavewallSpain

>Clade6CulturedFJ887905swalm1229springsedimentChina

>Clade6PsaturneaCulturedAJ252829IMSNU20052airGermany

>Clade6CulturedEU677789FXJ2021soilChina

>Clade6PpetroleophilaCulturedAJ252828IMSNU22072soilGermany

>Clade6ClonedEF516465FCPP410grasslandsoilCAUSA

>Clade6ClonedAB074634APe452aposymbioticaphidJapan

>Clade6ClonedEF540540M26oilshalewasteEstonia

>Clade6CulturedEF216350TFS575fjordsedimentNorway

>Clade6ClonedFM872941FB04H09floordustFinland

>Clade6CulturedFJ937942LS288marinespongeChinaSea

>Clade6ClonedGQ263688FW385CwasteIDUSA

>Clade6CulturedFJ817379YIM63233endophyteChina

>Clade6ClonedEF507108CZ52H03contaminatedsoilCzechRepublic

>Clade6ClonedFJ893767nbt35b02mouseskinUSA

>Clade6CulturedFJ214340YIM61043endophyteChina

>Clade6ClonedGQ263538FW299BwasteUSA

>Clade6PzijingensisAF3257256330TypeStrainsoilChina

>Clade6PzijingensisCulturedEU841604HBUM174915China

>Clade6PaurantiacaCulturedAF325727AS41537soilChina

>Clade7PchloroethenivoransCulturedAF454510SL1soil

>Clade7PyroSequFJ478886p8b10oksoilOKUSA

>Clade7ClonedEF589993A21pollutedriversedimentChina

>Clade7CulturedEF588213TrzetekiCC03040404workerPanama

>Clade7CulturedEF588226TrzetekiCC03010505workerPanama

>Clade8ClonedAM936575CM1D11contaminatedsoilFrance

>Clade8PspinosisporaCulturedAJ249206LM141TypeStraincavesoilSouthKorea

>Clade8ClonedEU979047g38rhizospheresoilChina

>Clade8CulturedDQ448726CNS139PL04marinesedimentPalau

>Clade8CulturedFJ948122MysmithiiUGM01040208Actino3worker

>Clade8ClonedEU527120zd430glaciersnowTibet

>Clade8PthermophilaCulturedAJ252830IMSNU20112compostSwitzerland

>Clade8PkhuvsgulensisspnovCulturedAB521672MN08A0297TypeStrainsoilMongolia

>Clade8CulturedFJ948118MysmithiiUGM01040103TMWB1workerlabnest

>Clade8ClonedDQ643700W4Ba36agriculturalsoilGermany

>Clade8DirectPCREU718355TrzetekiRMMA0501052841workerlabnest

>Clade8DirectPCREU718354TrzetekiRMMA0501052840workerlabnest

>Clade8DirectPCREU718334CywheeleriUGM0304290148workerlabnest

>Clade8ClonedAM935373AMGB8contaminatedsoilFrance

>Clade8CulturedFJ948123MysmithiiAGH01041701TMWB2workerlabnest

>Clade8CulturedJESSICA2TrseptentrionalisworkerfieldnestgardenTXUSA

>Clade9ClonedFJ661791PaAD11nitrateenrichedsoilMIUSA

>Clade9ClonedDQ643691W4Ba27agriculturalsoilGermany

>Clade9ClonedFJ568357A19YB03RMalpinesoilFrance

>Clade9ClonedFJ661792AaAC12nitrateenrichedsoilMIUSA

>Clade9PyroSequGQ002521nbu178d12c1humanskinUSA

>Clade9ClonedFJ570491A6YM19RMalpinesoilFrance

>Clade9PyroSequGQ099352nbw509f04c1humanskinUSA

>Clade9ClonedEU052164C3AA07savannahsoilTXUSA

>Clade9ClonedAM992500A44forestsoilOHUSA

>Clade9ClonedFJ568422A19YE18RMalpinesoilFrance

>Clade9ClonedAY921961AKYG1573farmsoilMNUSA

>Clade9PyroSequGQ062989nbw96c07c1humanskinUSA

>Clade9ClonedFJ616000F12C11agriculturalfieldMIUSA

>Clade9ClonedAY555622Act9sandsoilMDUSA

>Clade9ClonedDQ129564AKIW476aerosolTXUSA

>Clade9PasaccharolyticaY08536DSM44247TypeStrainwastegasbiofilterGermany

>Clade9ClonedEU132741FFCH12016prairiesoilOKUSA

>Clade10CulturedAF118130DB1refinerywastewaterGermany

>Clade10CulturedFJ711205KCITH6stalactitecavernAZUSA

>Clade10PbenzenivoransCulturedAJ556156B5soilGermany

>Clade10PhydrocarbonoxydansCulturedAJ252826IMSNU22140TypeStrainairGermany

>betweenClade9&10CulturedGQ924573ACT0146rootSolomonIslands

>betweenClade9&10PyroSequGQ021408nbu277g01c1humanskinUSA

>betweenClade9&10CulturedEU722525W101sourceunknown

>betweenClade9&10PyunnanensisCulturedAJ252822IMSNU22019TypeStrainsoilChina

>betweenClade9&10ClonedEU132625FFCH10433soilUSA

>betweenClade9&10ClonedGQ264114WC345wasteIDUSA

>betweenClade9&10CulturedFJ817406YIM63646endophyteChina

>betweenClade9and10PmongoliensisspnovCulturedAB521671MN08A0270TypeStrainsoilMongolia

>betweenClade9&10ClonedEF220405FI2FC12soilFalkland

>betweenClade9&10CulturedAF131481IM6071rainforestsoilSingapore

>ActinokineosporaenzanensisAB058395IFO16517

>ActinokineosporaterraeNR024774IFO15668

>CrossiellacryophilaNR024964NRRLB16238

>CrossiellaequiNR025088NRRLB24104

>KibdelosporangiumaridumAJ311174DSM43828

>KibdelosporangiumphilippinenseAJ512464DSM44226

>AmycolatopsisalbaNR024888DSM44262

>AmycolatopsisdecaplaninaNR025562DSM44594

>AmycolatopsiskeratiniphilaNR025563DSM44586

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